"Ovidius" University, Constanța
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Summary

Doctoral Thesis

Molecular investigations in esophageal cancer

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INTRODUCTION

Esophageal cancer is one of the most aggressive types of gastrointestinal(GI) cancers and because of the delayed diagnosis in our country caused by the poor socioeconomic status and the particular anatomic situation, approximately 70% of the patients are suffering of an advanced loco regional disease or with metastasis at unknown distances at the time of discovery. The patients are in general elderly, frail and malnourished, often cachectics. Its frequency remained constant in the last decade and despite the progress of surgical and oncological treatment, the mortality remains extremely high, because of the late diagnosis, the early metastasis and the frailty of the patients. It represents the fourth cause of death through cancer following lung neoplasm, colon neoplasm and prostate cancer. Even though in the last 15 years progress in the treatment of this disease has been noticed, there are still a lot of controversies regarding the optimal management of the condition.

Although, as mentioned above, the frequency of esophageal cancer has remained constant, a drop in the number of epidermis cancers has been registered and a rise in the number of adenocarcinoma cases. These two subtypes represent over 90% of the total of cases, the rest being rare tumors (melanoma, carcinoid, sarcomas, and lymphomas). The particular lymphatic drainage of the esophagus and the absence of the serosa explain the rapid and remote metastasis.

Surgery of the esophagus, as treatment of esophageal cancer presents special technical and tactical difficulties, in comparison with other organs because: its inaccessible position, its relations with vital organs of the chest and the need to mobilize the abdominal viscera for its reconstruction.

Cancer is a complex disease that occurs as a result of the gradual accumulation of genetic aberrations and epigenetic modifications that are beyond the control of normal cells. Neoplastic cells may have acquired numerous genetic aberrations: aneuploidy, chromosomal rearrangements, amplifications, deletions, gene recombination and mutations leading to loss or gain of functions. The aberrations lead to an abnormal behaviour common to all cancer cells: irregular growth, lack of contact inhibition, genomic instability and the likelihood of metastasis.

In esophageal neoplasm, the mechanisms responsible for the onset and evolution of the disease are far from being elucidated. In this context, research is directed towards identifying the molecular determinants of tumor induction and progression, in order to achieve an early diagnosis, preventive and predictive of disease progression, but also new, individualized treatment regimens.

In several types of cancer, biomarkers have improved our ability for a diagnosis, prognosis, treatment and prediction. In general, a suitable biomarker should be useful in defining and identifying risks in the early stages of carcinogenesis. In addition, biomarkers can be analyzed from a non-invasive perspective as well as from an economic perspective and, therefore, it is important to invest in the search of multiple biomarkers. Genes have been identified that are considered potential candidates for individualized therapeutic strategies aimed particularly against specific molecular targets.

The medical world manifests a keen interest for the introduction of some specific antitumor agents into current therapy (antibodies, inhibitors of growth factor receptors, antagonists of signal transduction factors, vaccines containing antigen cells) with or without chemotherapy.

Diagnosis and early treatment are efficient measures of reducing the mortality rate of esophageal cancer patients, but the majority of patients that are subject to hospitalization are in an advanced stage of the illness. One of the factors that is at fault for leading to this situation is that, no molecular marker present in the early stage is used in the diagnosis and treatment of esophageal cancer. Choosing different therapeutic measures depends largely on the clinical stage of the disease.

Esophageal cancer genetic changes have been reported as point mutations in the p53 and p16 genes, amplifications of the int-2, hst-1, cyclin D1 and EGFR, as well as loss of heterozygosity of chromosomal genes in many regions. However, the details of the molecular events that cause the malignant transformation of the esophageal epithelium are still unclear. Two classes of genes, proto-oncogenes and tumor suppressor genes, have a key role in the occurrence and development of cancer. Most cancers possess mutations in one or more inactivated proteins that normally operate to restrict the progression to the G1 stage of the cell cycle (e.g., Rb and p16). Studies have issued several theories that came to explain the malignant transformation seen as a disturbance of the normal biological cell cycle: the theory of genetic mutation, aberrant differentiation theory, the viral theory of cancel, cell selection theory.

For the practicing physician, a real progress in early detection and in determining prognosis, elements with a major impact on the survival rate of patients cannot be made at this time via the usual macroscopic investigation techniques. Progress in this area can be achieved by studying the molecular mechanisms of carcinogenesis, and by understanding the complex genomic and proteomic mechanisms leading to the emergence and development of cancer.

The present study aims to move to another level of treatment of esophageal neoplastic diseases, addressing genetic peculiarities and changes taken place to promote the disease. We wanted to show that each genetic change leads to a histological forms of cancer. Analyzing each of these forms we can obtain a protocol for early detection and individualized treatment based on the affected organ, the histopathological type of neoplasia and the existing genetically modifying neoplasia. Extending the concept, this protocol can be applied to their families to determine the genetic modification, for the purpose of early detection and treatment of the studied malignancies.

It is well known that early detection of genetic changes, that are accompanied by neoplasia, may lead to early treatment of their incipient form and thus increase the oncological patient's survival and his quality of life.

The paper is structured as follows: the general part begins with a brief overview of the anatomy of the esophagus, complete with data on physiology, morphology and pathology of the digestive segment, useful in understanding esophageal syndrome and mentions esophageal cancer genetics data; the personal and specialty part of the paper that includes the description of the material and the used methods of work, followed by the detailed results after the study has been completed.

This paper is the shy attempt of a surgeon to address aspects regarding research in this vast field of cellular and molecular biology, with direct implications in the treatment of malignant esophageal pathology.

I. GENERAL PART

Chapter I

THE ANATOMY OF THE ESOPHAGUS

The esophagus is a muscular cylinder, about 25 cm long, extending from the pharynx to the stomach, having its upper limit on the plane passing through the cricoid cartilage and its lower limit to the esogastric junction at the level of the opening of the cardia (4). The esophagus is divided into three portions: cervical, thoracic and abdominal.

The caliber of the esophagus is inconsistent with an average diameter of 2 cm and has *three esophageal straits* although not approved in NA, they have a great practical importance:

- *Superior* or *cricoidian* is located right next to the homonymous cartilage
- *middle* or *bronchoaortic* at the level where the esophagus is crossed anteriorly by the left bronchus and the aortic arch
- *inferior* or *diaphragmatic* at the passage through esophageal hiatus of the diaphragm (1);

Chapter II

THE STRUCTURE OF THE ESOPHAGUS

We've mentioned above that the esophagus is designed to link and to transport food from the pharynx to the stomach, and is topographically divided in three segments, each with its peculiarities (6, 9):

- Cervical, composed of striated muscle fibers;
- Thoracic, composed of striated and smooth muscle fibers. At this level, there are the following straits: cricoidian, aortic, bronchial and cardiac;
- Abdominal, composed of smooth muscle fibers. It extends from the esophageal hiatus to the cardia, underneath which we can find the diaphragmatic or inferior strait, determined by the contraction of connective tissue and smooth muscle fibers of the inner layer of muscular tunic of the esophagus (5, 6 ,9).

The esophageal wall consists of (9):

A. The tunica mucosa

- The mucosa is lined by squamous epithelium and its attached multi-layered nonkeratinized esophageal glands that secrete mucus (no actual role in digestion), thereby allowing the slipping of the food.
- In the abdominal portion of the esophagus, there are coated areas of gastric type epithelium, single ply cylindrical and even the gastric glands. The transition from multi-layered squamous epithelium to the gastric type epithelium takes place suddenly being objectified by the presence of esophageal mucosa lines separating gear red, paler and gastric red mucosa (5). This area can be the starting point of metaplasias and neoplasias of the esophagus (adenocarcinoma).

Barretts' esophagus is a columnar metaplasia of the esophageal epithelium more than 3 cm from the Z line, due to the prolonged aggression of the hydrochloric acid from gastro-esophageal reflux. Depending on the macroscopic appearance, there are 3 types: Barrett Island, Barrett "flame" and circumferential Barrett. From the microscopic point of view, can be metaplasias of intestinal epithelium (high risk of malignancy), junction type (like the epithelium of the cardia) or bottom (like gastric epithelium) (5). Positive diagnosis is objectified by the presence of goblet cells, which have morphological characters of hybrid between the columnar and squamous epithelium (5).

The Barretts' esophagus may progress to adenocarcinoma. Positive diagnosis can be difficult because dysplasia can handle only a limited area of the mucosa, endoscopic biopsy missing malignant degenerate areas (5).

- At the edge of the mucosa and submucosa we have lining muscle whose tone determines the stretch of the longitudinal esophageal mucosa.

B. The submucosa tunica

- At this level, there are loose connective tissue, a rich vascular network, submucose autonomous plexus (Meissner superficial and deep Henle) and the secretory glands of the esophagus.

C. The tunica muscle.

- With the exception that the upper part is so striated muscles and smooth at the bottom of the tunic the smooth muscle arranged in two layers: the internal format of the external circular muscle fibers, consisting of longitudinal muscle fibers.

The muscle neoplasms (sarcomas), the predominant effect on either of the two types of fibers lead to different developments: sarcoma longitudinal fibers extending in length and long evolution to dysphagia, as opposed to circular fiber sarcoma is accompanied by quick emergence dysphagia (9).

The particularity of the coats is the thickness of the two layers, in comparison with the rest of the digestive tract where the annular layer thickness. This reflects adaptation and specialization to transport food substances that have undergone transformations act only mastication.

Longitudinal fibers, shortening contractions, expand the esophagus before the bowel, which is pushed contraction ring, formed by the circular fibers of the bowl.

The fibers have longitudinal downward helicoidal trajectory, that at the bottom end in the circular layer.

D. The external Tunica

This is the peritoneum (above right) and retroperitoneal connective tissue (left rear) (9).

Chapter III

ESOPHAGEAL CANCER

Introduction

Esophageal cancer is a disease that requires rapid diagnostic methods in the early stages, and a therapeutic strategy to ensure the absence of relapse, a high level of comfort for the patient and increased life expectancy. There were discovered numerous genes involved in the development of esophageal cancer, but the mechanisms of action remain unknown. A number of new genes have been found and are potential candidates for determining prognosis and therapeutic strategies. An esophageal tumor model is necessary because it would help other researchers to channel diagnostic studies on genes of interest with the possibility of more rapid development of therapeutic means.

Cancer is a complex disease that occurs as a result of the progressive accumulation of genetic aberrations and epigenetic changes that manage to escape from the control of the normal cell (13). Neoplastic cells may have acquired many genetic aberrations (aneuploidy, chromosomal rearrangements, amplifications, deletions, gene recombination and mutations leading to loss or gain of function). Recent studies (13) have highlighted the importance of epigenetic alterations of certain genes that result in inactivation of their function in certain types of cancer. These aberrations lead to abnormal behavior common to all cancer cells: irregular growth, loss of contact inhibition, genomic instability and metastatic potential.

Analysis of mutations in oncogenes and tumor suppressor genes can demonstrate a specific association between these genes and the tumor type. These genes can be altered during carcinogenesis by different types of mutations such as point mutations, chromosomal translocations, deletions or gene amplification. Moreover, these genes can be analyzed at various levels - DNA, RNA, or protein (13).

Tumor cells exhibit a number of characteristics by which they differ from regular cells:

a) The do not depend on growth factors such as normal cells because they are able to secrete their own growth factors that stimulate proliferation, a process called autocrine stimulation, or as the receptors for growth factors from the surface are modified in such a way that the binding of growth factors is not necessary to stimulate the proliferation;

b) Normal cells require a space in order to increase the extracellular environment where tumor cells are anchored independently;

c) Normal cells respond to the presence of other cells and form a monolayer culture due to contact inhibition and tumor cells lack this aspect and often develops over or under one another;

d) The tumor cells are less adherent that the regular cells;

e) Normal cell proliferation stagnantes reaching a certain density, while the tumor cells continue.

Epidemiology and etiological factors

Esophageal cancer is a type of gastrointestinal malignancy with a poor prognosis, a modest and discreet installation. The disease predominantly affects older age groups with a peak incidence between 60 and 70 years; it is rarely encountered in children or young adults. The

indecency is higher in men with a sex ratio of 4 to 1. Until recently the most common type of esophageal cancer in the world was the squamous cell carcinoma. Adenocarcinoma is less than 15% of all cancers of the esophagus. Other malignant tumors of the esophagus, such as sarcomas, lymphomas, melanomas primary malignant and the small cell carcinoma are rare (14).

The incidence of esophageal cancer varies significantly depending on the geographic region and race. Its values may vary between regions of the same country, which demonstrates the important role of environmental or food related factors. Worldwide, the highest incidence of esophageal cancer is observed in Lixan, China, with over 130 cases per 100 000 inhabitants. Increased incidences of esophageal cancer are found in the regions of Iran, Russia, Colombia, and South Africa. In the Western Hemisphere, the incidence is approximately 5 to 10 cases per 100,000 inhabitants. In the United States, the estimated number of new cases of cancer of the esophagus for 2000 was of 12 300, with an estimated 12 100 death rate (22).

In the last two decades, both in the United States and in Western Europe there were changes observed to the common types of esophageal cancer. Thus the incidence of esophageal adenocarcinoma has increased, especially in male patients and that of squamous cell carcinoma remained unchanged or even decreased slightly. By the early 1990s, cases of adenocarcinoma have outnumbered the cases of squamous cell carcinoma and have become the most common type of esophageal cancer in men, accounting for about 60% of the cancers of the esophagus. This change in the epidemiology of esophageal cancer involves a number of factors which cannot be explained in a simple reclassification of gastric carcinoma in the esophagus or cardia adenocarcinoma as justifiable increase in the number of cases of Barrett's esophagus (40).

Etiology

The most important predisposing factors of esophageal cancer in developed countries, under both numerous studies, are smoking and alcohol consumption (Table 1) (14).

Squamous cell carcinoma	Excessive smoking The consumption of too much alcohol Family history of cancer developed in the head or neck History of radiation Chronic esophagitis (the most common being in Asia and Africa) Chronic stricture (ingestion of chemicals, iradiation) Tylose (palmar and plantar hiperkeratinization) Plummer-Vinson syndrome Achalasia Diet / Nutrition Deficiency in carotene, vitamins C and E, riboflavin, selenium and zinc Low consumption of fruits and vegetables Increased consumption of red meat and foods with nitrates Consumption of hot beverages
Adenocarcinoma	GERDandBarrett's esophagus Obesity Smoking Alcohol Scleroderma Family historyof colon cancer Medication: β-agonists and tephilina (prolonged use> 5 years)

Table 1. Risk factors for esophageal cancer.

While for squamous cell carcinoma the most important risk factors are alcohol and tobacco, the main risk factor for adenocarcinoma of the esophagus is the Barret. Barrett's esophagus, a premalignant known lesion, is a consequence of the chronic gastro esophageal reflux (GERD) in which the squamous epithelium of the distal esophagus is replaced by columnar epithelium of intestinal type (41).

In some regions of the world, the very large number of cases of cancer of the esophagus has been assigned to other factors such as those related to the environment and diet and nutrition. These include ingestion of hot food and fizzy drinks, canned foods containing nitrates, deficiency of essential vitamins (carotene, riboflavin, vitamin C and E) and minerals (zinc and selenium), as well as unbalanced consumption of fruits and vegetables . HPV virus is also the potential rise of squamous cell carcinoma of the esophagus (12).

Colon cancer and breast cancer may be associated with an increased risk for cancer of the esophagus. In fact, cancer of the colon is associated with the adenocarcinoma, whereas the breast cancer is associated with both adenocarcinoma and squamous-cell carcinoma of the esophagus. The increased risk of squamous cell carcinoma of breast cancer is higher in patients who underwent radiotherapy as part of treatment. Irradiation can destroy genetic defenses or cause chronic oesophagitis and oesophageal narrowing, both predisposing to the development of squamous cell carcinoma (14).

Improving the current methodology for diagnosing esophageal cancer through early detection and treatment of mini-invasive lesions individualized has as final result the increase survival and improvement of the quality of life of these patients by reducing morbidity and mortality. Results of the studies will allow the identification of gene markers value that will be useful in designing new individualized treatment regimens in the diagnosis and follow-up of applied therapy and monitoring tumor progression.

The differential diagnosis of esophageal cancer

Diagnosis is made with the following conditions:

a. Benign esophageal stenosis: gastroesophageal reflux disease, stenosis postcaustic, postradica esophagitis. Anamnesis diagnosed with barium swallow, endoscopy with biopsy acknowledges.

b. Esophageal diverticulum, diagnosed by barium transit.

c. Achalasia, accompanied by dysphagia, liquid food first and then the solid weight loss. To establish the diagnosis laboratory tests will be made, barium examination, endoscopy to visualize the lesion and microscopic sample will be taken. It will also check the closing pressure of cardia and esophagus by esophageal manometry propulsion.

d. The presence of extrinsic compression: mediastinal tumor, lymph nodes, lung hydatid cyst or tumor, goiter, plunger, aorta aneurysm.

Chapter IV

SURGICAL TREATMENT

The purpose of the oncological surgical intervention for this medical condition is the resection of the primary tumor and of the drainage lymph nodes, and it represents the golden standard in the treatment of this ailment.

Esophagectomy

Esophagectomy is the main therapeutical attitude, which is however an aggressive intervention with major risks. In patients with a resectable tumor, showing no signs of loco-regional adenopathies, metastases or contraindications (cardiorespiratory afflictions (17) and denutrition(18)), surgery is the primary option (19).

The choice of the surgical procedure depends on several factors:

- anatomical location of the lesions, where the cervical positioning is encumbered by technical difficulties and consequently palliative interventions are practiced, while the lesions located in the lower half and third of the esophagus may benefit more often from curative interventions, the lesions with cervical location are extremely aggressive cancers because of the very intensive cervical lymphatic system (20);
- the local-regional and remote spreading of the lesions, with graduation of the lesions by stages;
- the surgical procedure employed for restoring of the transit, frequently involving the stomach, somewhat more seldom the colon; with pros and cons for each of them.

A correct transthoracic approach can be achieved by means of an anastomosis in the upper thorax in case of smaller tumors, and of a GEJ procedure (Ivor-Lewis method), or in the throat in case of larger lesions. The transhiatal approach avoids a thoracotomy and places always the anastomosis in the throat. The distal esophagus resection and its reconstruction may be also approached via the left side of the chest or by means of a thoraco-abdominal approach (21).
Esophagectomy is a radical resection, where a tissue mass with 10 cm borders proximal and distal from the tumor includes the thoracic esophagus, the thoracic duct, the azygos vein, the posterior pericardium, and the soft tissue in the posterior mediastinum.

The stomach is used frequently for the reconstruction of the esophagus. It is easily mobilised, it enjoys an excellent vascular input, and in almost every case it reaches up to the throat and the root of the tongue. The new esophagus can be placed either in transthoracic or retrosternal position. When the stomach cannot be used because of a previous gastrectomy or of a tumor, it is possible to use the large bowel for reconstruction. Frequently, the left colon is used with vascularisation based on the reliable ascending branch of the left colic artery. With the three anastomoses required for restoring the gastro-intestinal continuity, there is a higher degree of morbidity, but the functional results are excellent (23).

Patients with metastatic disease or with tumors in an advanced stage are treated with palliative intentions. The palliative care means should be directed first of all to the improvement of the dysphagia and of the esophageal obstruction. This can be achieved by several means, including through palliative radiations, by endoscopic dilatation, by endoscopic stenting through prosthetics under general i.v. or local oro-pharingeal anesthesia, with insertion of a prosthesis

of synthetic/composite material, or a selfexpandable metal stent, sliding along a metal wire as a guide, under radiological guidance, endoscopic Laser therapy, or another light-based therapy (for instance, photodynamic therapy). Most efficient proved to be the selfexpandable metal stents, which keep their position in time and also along with the size increase of the tumor, one of their qualities being the maintaining of the permeable lumen. Prostheses may not be used in case of tumors with pharingeal-esophageal location, or of those located in the thoracic esophagus, but which generate multiangularstenoses, or in case of esocardialtumors. Palliative chemotherapy has only a limited role in these circumstances and just a marginal impact on the patient's survival.

AIMS OF THE SURVEY OF THE DOCTORAL DISSERTATION

The main purpose of the survey is the early tracking of the cancer of the esophagus through up-to-date methods, meaning the contribution of the genetic changes in this disease.

Certainly, this is necessary because the esophageal syndrome and particularly the dysphagia are revealed clinically late, and the screening for the esophagealtumors is not practiced in Romania. This situation could be bettered by the use of certain tumoral markers in view of determining a positive and differential diagnosis. A great prognostic importance has also the histopathologic type, since the neoplasms of the lower esophagus are quite often in fact adenocarcinomas which do not respond to adjuvant treatments, and the cancers of the upper esophagus (of ectodermal origin) are often epitheliomas or squamous carcinomas, with a better prognosis and a better response to the adjuvant treatment.

At present, molecular investigations are being conducted all over the world in cancer cases, with the hope to set up the coding of certain genetic treatment protocols.

Our survey refers to the genetic markers of the cases of esophageal cancer.

2. PERSONAL RESULTS AND DISCUSSIONS

Chapter V

ANALYSIS OF THE GENERAL CLINICAL DATA

Distribution by age groups

This survey included patients with ages ranging between 42-77 years, as follows:

2	between 40 and 50 years
10	between 50 and 60 years
8	between 60 and 70 years
3	over 70 years

Distribution by gender

The group was divided into 19 cases in males and 4 cases in females.

Background of origin

There is an increased incidence of esophageal cancer in patients coming from the urban area as compared to the rural one: 17 from towns and cities and 6 from villages.

Risk factors

Alcohol and smoking represent proven risk factors in the increase of the incidence of the esophagus cancer, particularly of the proximal one. Alcohol impacts the absorption of folates, reducing their bioavailability, and smoking increases 2-3 times the risk of cancer occurrence, as compared to non-smokers. From the 23 patients diagnosed with esophageal cancer, 19 patients are smokers and 4 non-smokers. From among them, 17 male patients and two female patients were smokers, and their distribution by their place of residence (urban/rural), the male patients numbered 10 from the urban area and 7 from the rural area, while the two smoking female patients came from urban backgrounds. The distribution by age groups of the smokers is relatively equal, with a slight increase in the age span comprised between 60-70 years. As for the alcohol consumption, we encountered 19 patients who declared to ingest ethanol, out of which there were 17 men and two women. Considering their background of origin, 8 men and one woman came from urban areas, while the remaining 9 males and one female came from rural zones

Diagnosis of esophageal cancer

The clinical diagnosis of esophageal cancer is obtained based on the presence of the esophageal syndrome, mainly based on the occurrence of the progressive dysphagia, initially for solids, followed by dysphagia for liquids, accompanied by important weight loss and even cachexia in the absence of supportive treatment by drip-feeding, or of practicing gastrostomy for feeding the patient. Most of the patients came too late to the physician's, mostly with a dysphagia in the 3rd or 4th degree, more seldom in the 1st or 2nd degree. Number of patients by how critical their condition was: 1st degree – 2 patients, 2nd degree - 4 patients, 3rd degree – 8 patients, 4th degree – 9 patients.

Esophageal pain is present in form of pyrosis in 4 patients, as odynophagia in 4 patients, as retrosernal pain in 10 patients, and installed in other locations in 3 patients.

Besides, the patients complained about regurgitation, sialorrhea, haemorrhages, and they showed also signs of neoplastic spread (adenopathies, irritative cough associated or not with hoarseness, bone pains and weight loss).

Location of tumors

The prevailing location of the esophageal cancer in our investigation group can be found in the lower third for a number of 10 patients, followed by the middle third in 8 patients and the upper third in 5 patients.

Analysis of the histopathological type

From microscopic view, the 23 patients of our investigation group showed the following types of cancer: 17 had squamous carcinomas, out of which 9 were spinocellular carcinomas, 5 were carcinomas with fusiform cells and other 3 squamous carcinomas of the adenoid cystic type and 6 adenocarcinomas divided into 3 adenocarcinomas of the diffuse mucinous type and 2 adenocarcinomas with signet ring cells and 1 adenosquamous carcinoma.

From macroscopic view, among the 17 squamous carcinoma we could distinguish the following types: 6 were ulcero-vegetantetumors, 4 ulcerating tumors, 3 infiltrative (stenosing) tumors, 3 ulcero-infiltrative tumors and 1 verrucous lesion, while the 6 adenocarcinoma featured the following types: 3 ulcerative, 2 polipoid-vegetante and 1 infiltrative tumors.

Distribution by TNM stages

From an analysis of the investigation group by TNM stages it resulted that most of the patients were present in the stages III and IV.

Stage TNM (whole group)	Number of pacients: 23	Percent %
Stage I (T1N0M0)	1	4%
Stage IIA (T2N0M0)	1	4%
Stage IIB (T1N1M0)	2	9%
Stage IIB (T2N1M0)	2	9%
Stage IIA (T3N0M0)	2	9%
Stage IIIA (T3N1M0)	3	13%
Stage IIIC (T4N0M0)	2	9%
Stage IIIC (T4N1M0)	3	13%
Stage IV (T4N1M1)	4	17%
Stage IV (T3N0M1)	3	13%

Chapter VI

RESULTS AND DISCUSSIONS

Highlighting of molecular changes on the level of oncogene activation (immunofluorescence assays);

In view of conducting this action, two molecular markers (oncogenes) were earmarked, namely HER2 and EGFR, which were analysed by immunofluorescence in 5 samples of tumoral tissue and 5 samples of normal tissue of the esophagus.

HER2/neu (also known as **ErbB-2**) is the codification genefor the "Human Epidermal growth factor Receptor 2". It is a member of the ErbB protein family, and in fact a tyrosine-kinase involved in the signalling routes for the cellular growth and differentiation. It is a proto-oncogene located on the longer chromosome branch 17 (17q21-q22). Most of the studies are made on breast cancer cases (24).

The supraexpression HER2 was reported only in 7.7 % of the esophagus carcinoma cases and in only one of the 6 cases of esophagus adenocarcinoma. Activation of the *Neu* increases the motility of the tumoral cells, the protease secretion, the invasion and it modulates also the *checkpoint* function of the cellular cycle.

In the investigated esophagus cancer cases, a supraexpression of HER2 (fig.2) is highlighted, as compared to the normal tissue specimens coming from the same patient (fig.1). In fig. 7-8 there are shown immunofluorescence images on the esophageal tissue coming from patient 5. HER2 has a continuous perimembranous disposition.

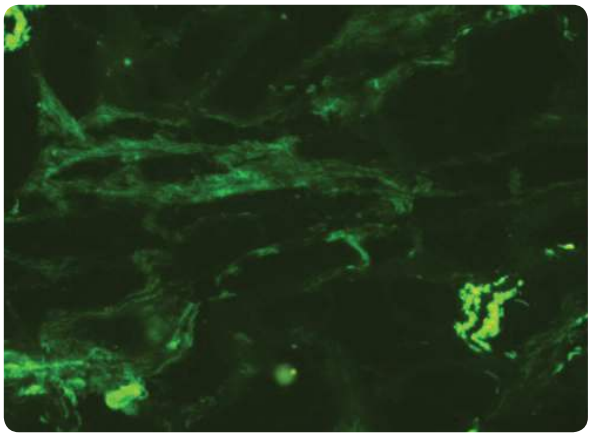

Figure 1. Immunofluorescence for HER2/neu in normal esophagus tissue.

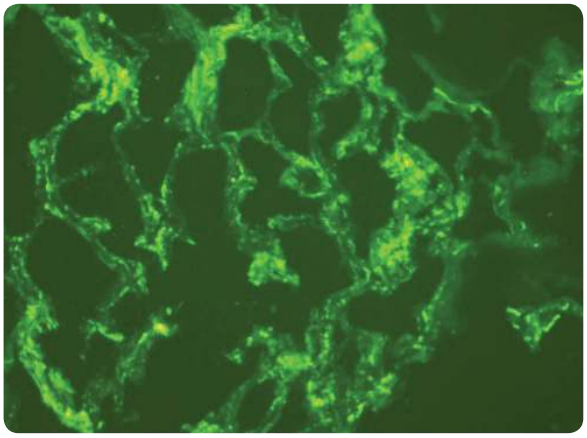

Figure 2. Immunofluorescence for HER2/neu inesophagus cancer.

The **EGFR** (epidermal growth factor receptor), also called ErbB-1; HER1 in humans, is a superficial receptor, which belongs to the family of receptors of the epidermal growth factors (EGF-family), which have as ligands the extracellular proteins (36). It is closely related with the tyrosine-kinases receptors: HER2/c-neu (ERB2), HER3 (ERB3) şi HER4 (ERB4). In cancer, the EGFR gene features mutations which affect both the expression and its activity (37).

The EGFR is present on the surface of the cells and it is activated by its binding to specific ligands, including epidermal growth factors and TGFα. Activation of EGFR implies the transi-

tion from the monomer to the homodimer form and, by the binding of other members of the ErbB family, such as Erb2/Her2/neu, it passes to the heterodimer form. Dimerisation of EGFR stimulates the intrinsic activity of the protein tyrosine kinases, which has as a result the autophosphorylation of some tyrosine remnants (Y) in the C-terminal domain of EGFR. Among them - Y992, Y1045, Y1068, Y1148 and Y1173. Such autophosphorylation determines the downstream activity and the signalling of other proteins with phosphorylated tyrosines (with an SH2 binding domain of the phosphotyrosine). They initiate signalling cascades: MAPK, Akt and JNK, which lead to the DNA synthesis and cell proliferation. These proteins modulate the migration, adhesion and proliferation of cells.

Mutations, boosting and expression disorders of the EGFR or of other members of the family it belongs to are described as implications in 30 % of the total number of epithelial cancer cases (25). The identification of EGFR as an oncogene leads to the development of certain directly targeted therapies against cancer. This is how Gefitinib (26) and Erlotinib appeared for lung cancer, and Cetuximab for colon cancer. Cetuximab and Panitumumab are used as inhibitors of monoclonal antibodies. The monoclonal antibodies are barring the binding domain of the extracellular ligands; when this situs is blocked, the signal molecules are not able anymore to attach and cannot activate the tyrosine kinases any more.

The EGFR supraexpression is a genetic alteration frequent in pre-malignant dysplastic lesions of the squamous esophagus and also a genetic change that occurs in the early stages in the cancer cells of the squamous esophagus (27, 28).

The continuous perimembranous disposition of the EGFR protein and the positive reaction of the tumoral tissue of the esophagus to the treatment with EGFR antibody are also highlighted in case of the investigated patients (fig. 4). In case of normal esophagus tissues, the immunofluorescence reaction was negative (fig. 3).

The immunofluorescence technique enables us to obtain data which may allow us to estimate the coloration continuity along the membranes of the tumoral cells, as well as the number of immunocoloured cells, which will be useful to the oncologist in the treatment against EGFR.

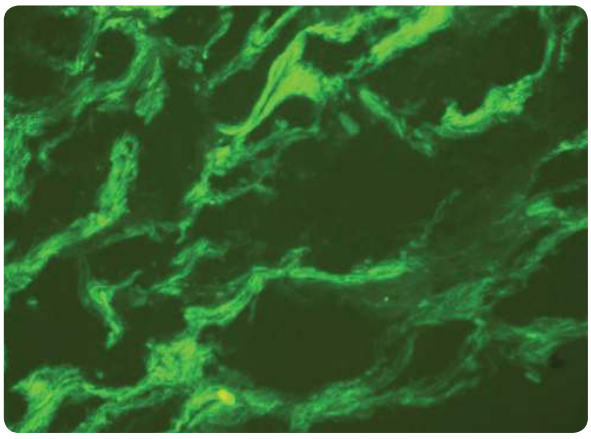

Figure 3. Immunofluorescence for EGFR in normalesophageal tissue.

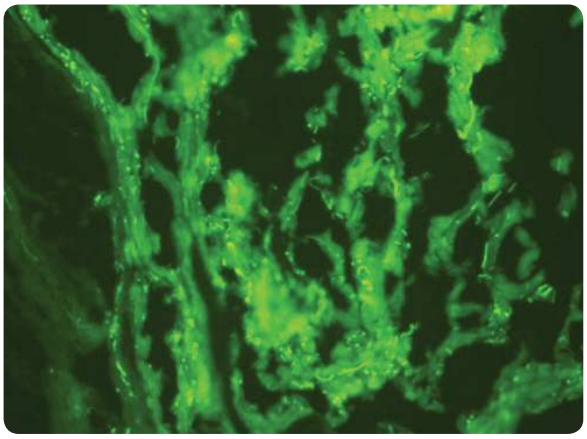

Figure 4. Immunofluorescence for EGFR in case of a patient with esophageal cancer, included in our survey.

Highlighting of molecular changes on the level of inactivation of the tumor suppressor genes (immunofluorescence surveys)

In view of conducting this action, two molecular markers (tumor suppressor genes) were earmarked, namely APC and p53, which were analysed by immunofluorescence in the cases of tumoral tissue and normal esophagus tissue.

Adenomatous polyposis coli (APC), also known as **deleted in polyposis 2.5** (DP2.5), is a protein codified by the gene APC, classified as a tumor suppressor gene. It is involved in the pre-

vention of out-of-control growth of the cells, as well as in cell division: it provides the right number of chromosomes during cell division. The activity of beta-catenin is controlled by APC via the Wnt signalling way. Through the regulation of beta-catenin, the genes are inhibited which the cell division and prevent the cell invasion (29).

Adenomatous familial polyposes (FAP) are caused by mutations in the APC gene. Most of these mutations lead to the production of abnormally short and non-functional APC protein. The abnormal APC proteins cannot suppress the continuous cell growth, fact which will lead to the formation of polyps, which can turn into cancerous growths.

In figure 5 there is shown an immunofluorescence image taken on normal esophagus tissue (remote from the tumor), with a negative APC protein expression, whereas in figure 6 there is shown an APC supraexpression in tumor tissue. Both images are taken on tissues coming from the same patient diagnosed with squamous esophagus cancer.

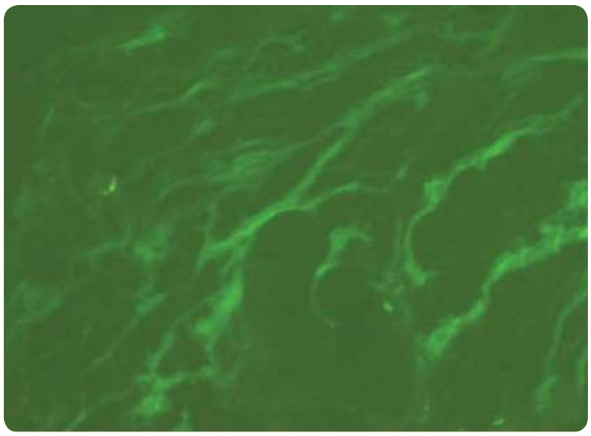

Figure 5. Immunofluorescence for APC in normalesophagus tissue.

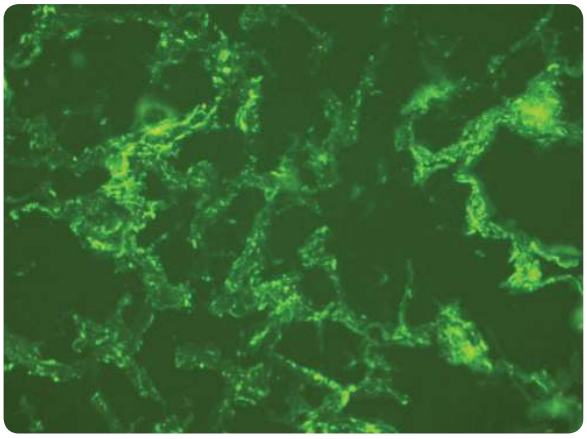

Figure 6. Immunofluorescence for APC inesophagus cancer.

Identification of possible genetic changes on the level of the control process of the cellular cycle (immunfluorescence surveys)

The analysis of mutations on the level of the genes known as „mitotic checkpoint" leads to the loss of gene functions. The mutations on the level of the gene p53 range among the best known genetic lesions described in cancer. P53 operates in a homotetrameric complex as a transcription factor which induces the genetic expression and which can facilitate the cessation of the cellular cycle, the DNA repair and the apoptosis.

One single mutant p53 protein in the tetrameric p53 complex is enough to cancel the normal function of the entire complex. Moreover, the mutant p 53 proteins have a longer half-life than the wild-type p53 proteins (with no mutation), fact which has a direct impact on the accumulation of mutant p53 proteins in the cell and thus the activity of the normal p53 will be inhibited. The increase of the intracellular p53 concentration can be highlighted by immunohistochemistry. A great number of immunohistochemistry studies stress the fact that more than 50% of the esophageal adenocarcinomas feature a marked p53 supraexpression (38, 39).

The p53 supraexpression detected in the cases investigated by means of immunofluorescence (figure 8) is related to the prognosis, degree of differentiation, degree of proliferation, and it is sustained by the number of immunomarked cells and by the intensity of the marling. In figure 7 there is shown an immunofluorescence image taken on normal esophagus tissue de (remote from the tumor), with a negative p53 protein expression.

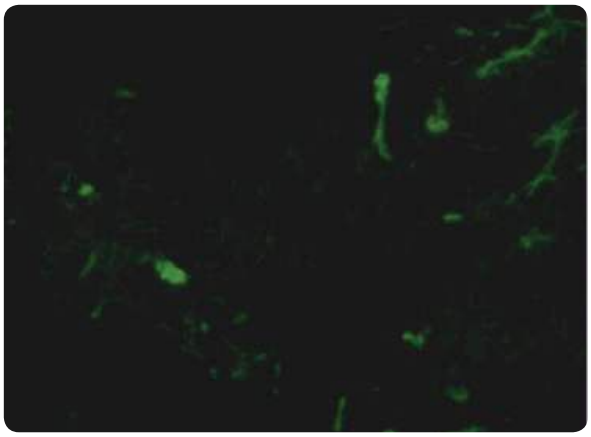

Figure 7. Immunofluorescence for p53 in normalesophagus tissue.

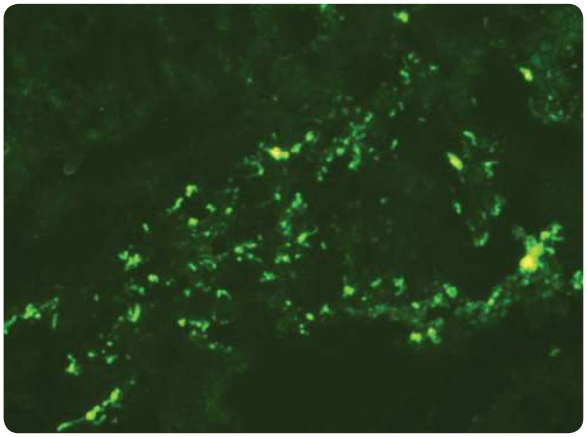

Figure 8. Immunofluorescence for p53 in esophagus cancer.

Assessment of the apoptotic capacity of the cells in different stages of esophagealtumor (immunofluorescence assays)

The normal cell populations which get divided maintain a balance between the cell proliferation and the cell loss, which is an important fact in maintaining a constant number of cells in the tissue. When there is an increase of the cell proliferation and a decrease of the apoptosis, or both, an out-of-control cell growth occurs, which will result in the formation of a tumor.

The apoptosis or programmed cell death is a mechanism responsible for the loss of cells. The apoptosis is a mechanism which causes the removal of cells grown old or defective (which might interfere with the normal functions of the cells or will cause neoplastic proliferation) and the DNA repair. The apoptosis can be detected by means of immunohistochemistry techniques which highlight the DNA fragmentation. An increase of the apoptosis rate has been highlighted, with an increase of the seriousness of the histological modifications in intestinal metaplasia/ dysplasia and in carcinoma, and also in the increased degree of Barrett dysplasia and adenocarcinoma (30, 31, 32).

The Bcl-2 proto-oncogene codifies for a protein inhibiting the cell apoptosis. Bcl-2 expression was found to be increased in cases of reflux esophagitis, nondysplastic Barrett esophagus and low-degree Barettesophagus dysplasia, at rates of 70-100%. The Bcl2 expression was found to be decreased, even absent in cases of carcinoma (0-40%) and high-degree dysplasia (0-25%), (32,33). Apparently, the apoptosis inhibition by supraexpression of the Bcl-2 protein seems to occur in early evolution stages of the Barrett dysplasia towards carcinoma, having as a result the extension of the survival rate of the cells and the promotion of the neoplastic progression.

In our research works aimed to highlight apoptosis, by watching the Bcl2 expression level, we could not find any alteration of the expression of this protein in the tumoral tissue of the patients with esophagus cancer, as compared to their normal tissue (fig.9-10). This allows us to conclude that, in cases of malignant esophagealtumors processed with different degrees of differentiation and different invasion stages, there is no Bcl2 expression, since the apoptotic capacity can be noticed especially by means of post-treatment methods and techniques. (The apoptotic capacity is noticeable in patients with esophagus cancer after they have undergone anti-cancer treatment.)

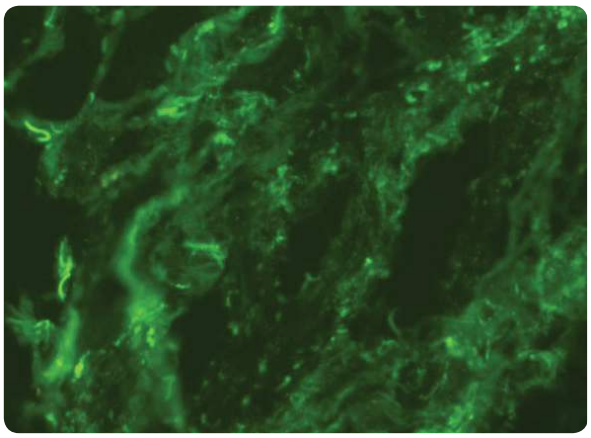

Figure 9. Immunofluorescence for Bcl-2 in normalesophagus tissue.

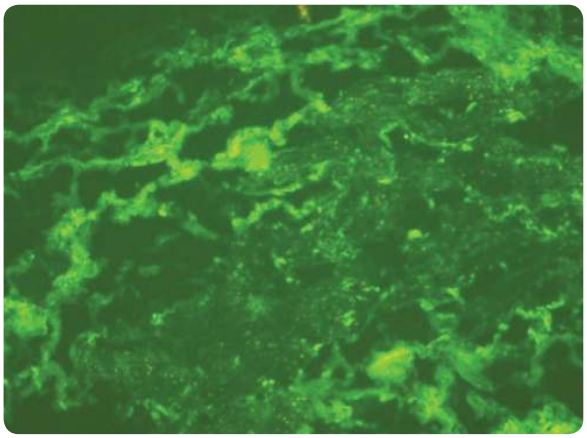

Figure 10. Immunofluorescence for Bcl-2 in esophagus cancer.

RESULTS OBTAINED FOLLOWING THE ANALYSIS OF DELETIONS AND DUPLICATIONS BY MEANS OF THE MLPA METHOD

In order to be able to make an accurate reading of the results of an MLPA analysis we shall consider several aspects. In fact, the analysis starts from the unprocessed genetic profile, resulted following the migration and separation of the amplification products, on the genetic analyser ABI Prism 310. Through its processing by means of the programme „GeneMapper ID v3.1", the genetic imprint of the individuals will be obtained.

A more precise examination of the obtained profiles can be carried out in a modified Excel programme, called Coffalyzer. In the beginning, the values for height and corresponding area of every peak of a single passing, of a single patient, resulting following the processing in the programme GeneMapper ID v3.1, are exported in an Excel document and only afterwards introduced into the Coffalyzer programme. This programme allows the normalization of the fragments separated through MLPA, and presented as a ratio of the number of copies. This ratio is the result of dividing the area of each peak to the sum of the areas of all the peaks of an investigated specimen. The relative ratio of the area of each peak is then compared with the one obtained on the DNA control specimens, coming from the patients whose BRCA2 gene has not been modified.

Also, within the frame of one experiment it is necessary to process at least three DNA control specimens, as the Coffalyzer programme sets up an internal statistic, too, which allows a better levelling of the control peaks.

Following this levelling, the ratios with values ranging around 1 (0.8 – 1.2) are deemed not to have suffered any alteration of the deletion or duplication type. It is classified as a deletion when the ratios are lower than or equal to 0.5; and respectively as duplication when the ratios are higher than or equal to 1.5. Moreover, for deletions there are accepted even values of 0.6 – 0.7, while for duplications there are accepted the values of 1.4 – 1.3, which is in compliance also with the loss percentage of 35 – 40%.

There have been investigated 5 patients with the MLPA method, in view of highlighting certain possible correlations between the chromosome mutations existing in the BRCA2 gene (deletions or amplifications) and the esophagus cancer.

The analysis of the MLPA results begins from the not processed genetic profile resulting following the migration and separation of the amplification products on the genetic analyser ABI Prism 310. By its processing by means of the programme „GeneMapper ID v3.1", the genetic imprint of individuals is obtained in the end (Figure 2).

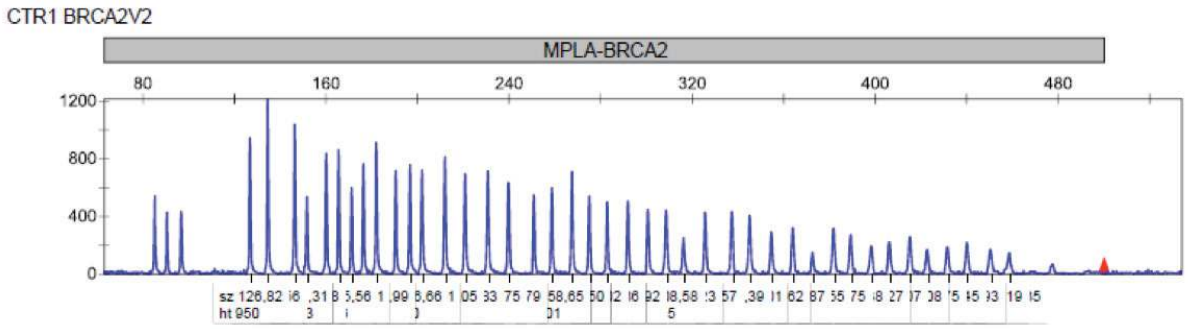

Figure 2. MLPA chromatograms in case of control specimens for the investigated gene BRCA2.

Ten control specimens were used for the normalizing of the results. In table 4 there is shown the genetic profile of the gene BRCA2 in case of each of our patients.

All ten patients have shown at least one deletion- or amplification-type modification in the gene BRCA2. A possible correlation is to be noticed between the presence of certain deletions of the exons 4 and 5 and the esophagus cancer, which modification appears in all the ten investigated patients. Other deletions which may be related with the development of the esophagus cancer were highlighted in the exons 23, 24 and 27A in case of six out of the ten investigated patients. Amplifications that might be correlated with the presence of esophagus cancer are encountered in the exons 8 and 10 in six of the ten analysed patients.

ANALYSIS OF THE MUTATIONS IDENTIFIED IN THE GENE P53 IN PATIENTS WITH ESOPHAGUS CANCER

In view of the identification of polymorphisms in the gene p53 for the cases of esophagus cancer included in our survey, a series of PCR reactions were carried out with the designated sets of primers. Amplifications were carried out on tumoral tissue specimens. The amplicons were verified later on by electrophoresis in 1.7%, agarose gel, and from there the following electrophoretic profiles were obtained.

Patient	Tumor stage l	Polymorphisms	Modified amino acid
6	IIa	codon 213 CGA → CGG	Arg → Arg
7	Ib	codon 175 CGC → CAC	Arg → His
8	I	intron 7 + 18437, T/C +18457, T/G	-
11	IV	codon 220 TAT → TGT	Tyr → Cys

Table 2. Mutations in the gene p53, identified in patients with esophagus cancer in various stages of the tumor.

The purified PCR products were sequentiated by means of the genetic analyser ABI 3130 (Applied Biosystems). The sequences obtained both from tumoral tissue specimens and from blood were compared with the sequence in p53 included in the GenBank data base. There were identified a great number of nucleotidic polymorphisms. Out of ten analysed patients, four (40%) featured polymorphisms in the gene p53.

In the exon 5, a polymorphism was noticed in patient nr. 7. This polymorphism corresponds to a punctiformheterozigote missense mutation located in the codon 175 (CGC → CAC). The normal codon codifies for arginine, the modified codon codifies for histidine. Both of them are polar amino acids and, as a consequence of this mutation, the polarity of the protein is not affected.

In the exon 6, two punctiform heterozygote mutations were identified: i) a silent one in the codon 213 CGA → CGG; this mutation was found both in the tumor specimen, and in the blood of the patient nr. 6, and the amino acid codified by both codons is arginine; ii) a missense mutation in the codon 220 TAT → TGT, in the tumor specimen of our patient nr. 11; the codon TAT codifies for thyrosine, and the codon TGT for cysteine. Cysteine and thyrosine are neutral amino acids, but cysteine is a weakly polarised amino acid, whereas thyrosine is not. This latter modification identified in the exon 6 can influence the polarity of the protein. Other genetic modifications are highlighted in the investigated gene p53; in this case we are speaking about polymorphisms in the intron 7 of the gene, identified in patient nr. 8. The two polymorphisms were noticed in the positions +18437, T/C and +18457, T/G.

The correlation of the genetic data with the clinical data of the patients allowed us to notice that the modifications in the gene p53 appear in early stages (I-II) of the squamous esophagus cancer. The more advanced cancer stages can be associated with modifications of the protein polarity, which could lead to changes of the protein conformation and to the alteration of its response capacity at the tumor level (table 2).

The mutations in the gene p53 (polymorphisms, deletions) are among the most common genetic modifications discovered in human cancer cases. More than 85% of the p53 mutations discovered in cancer cases are missense mutations. In case of certain types of cancer, this type of mutations is associated with advanced tumor stages and with a low rate of survival (34,35). The missense mutations produced in vivo may lead to substitutions of a single amino acid, which may result in changes in the p53 protein conformation. The consequence of such modifications may be their contribution to the progress of the tumor and to an unfavourable prognosis of the disease (35).

DISCUȚII

It is very difficult to detect a cancer case in its preclinical stage, and most such cases are discovered too late, when dysphagia becomes clinically obvious and tumors can be noticed imagistically (usually by an endoscopic investigation and by CT).

The tumor stage is advanced (stage III and, more frequently stage IV) because, from both clinical and paraclinical view, the patient is brought in too late (poor health education, deficient relation between patient and family doctor, extension of the treatment for the gastroesophagealreflux despite not conducting any digestive endoscopy, which could help to determine the diagnosis of esophageal cancer). In the infraclinical stage, with minor clinical signs, or even in the absence of imagistic signs, molecular investigations are extremely useful, because they help detecting the group of patients who show genetic modifications that can lead to the occurrence of esophagealneoplasia (just as the women suffering from breast neoplasm are candidates to develop a tumor in their contralateral breast, too). We consider that such genetic tests with a very high accuracy degree are significant and truthful for the esophageal cancer; this means that the genetic investigation is decisive in the field of incertitude of the quick diagnosis of esophageal cancer, in the "border-line" area. The major advantage of molecular investigations leads to surgical interventions in the stage of oncological safety. Because of the positioning of this organ in three zones (throat, thorax and abdomen), the esophageal cancer features a lymphatic spreading in all these areas and becomes rapidly inoperable, since the lymph node metastases and the local invasions develop in an inoperable (difficult to approach) mediastinum zone.

The second major importance of the molecular investigations consists in the execution of the individual treatment of the neoplasias. The classical treatment implies its execution in relation with the affected organ and the histopathological type of that cancer case. Depending on these parameters, there are permanently updated standardized protocols for the chemo- and radio-therapy.

Chapter VII

CONCLUSIONS

1. Cancer is a complex disease which appears as a result of a progressive accumulation of genetic aberrations and epigenetic modifications which manage to elude the normal cellular control. The environment factors play also an important role; it is believed that approximately 60-90% of the cancer cases are believed to be caused by environment factors. The neoplastic cells can have numerous acquired genetic aberrations (aneuploidy, chromozomal rearrangements, amplifications, deletions, genetic recombinations, and mutations which lead to the loss or acquisition of a function). Aberrations lead to an abnormal behaviour common to all neoplastic cells: irregular growth, lacking contact inhibition, genome instability and probability to develop metastases.

2. In several types of cancer, the biomarkers have improved our capacity to set a diagnosis, a prognosis, a treatment and prediction. Generally, an appropriate biomarker should be useful in the definition and identification of risks in the first stages of carcinogenesis. Moreover, the biomarkers can be analysed in a non-invasive and economic manner and, consequently, it is worthwhile to invest in the research meant to find more biomarkers, through the emergence of new science domains, such as genomics and proteomics. This allows the study of all the genes in a cell or in an organism and the analysis of a number of proteins.

3. The analysis of the clinical parameters enables us to draw the following conclusions: an increased incidence of esophageal cancer in the male gender, with a maximum in the decade of 50-60 years of age, with smoking and alcohol consumption being the most important risk factors (particularly when they are associated). The urban zone of origin of the patients and the location of the tumor in the lower third are prevailing. Adenocarcinomas are encountered more often than squamous carcinomas, and they are frequently located in the lower half of the esophagus, while the squamous carcinoma occurs more frequently in its upper half. The patients called on the physician in a higher percentage in the stages III and IV of the disease, more seldom in stage II, and very seldom in stage I of the tumor development.

4. Surgery of the esophagus displays important technical-tactical difficulties, as compared to other organs, because of it is placed in a location hard to reach and because of its relations with various vital organs, as well as because of the need to mobilise the abdominal internals in view of its reconstruction, and it seems that the esophagectomy with or without lymphadenectomy, associated with chemo-radio-therapy, is the best therapeutical solution. (15)

5. Selection of the surgical approach technique depends on several factors, such as: the anatomical location of the lesion, the cervical location being afflicted by technical difficulties, and there are frequent cases when palliative interventions are practiced, while the lesions situated in the lower half and third of the esophagus may benefit more often from curative interventions; the local-regional and remote spreading of the lesion; the surgical procedure applied for restoring the transit, frequently involving the stomach, more seldom the colon, for either of them speaking different pros and cons and, last but not least, it depends on the possible contraindications (cardiorespiratory afflictions and denutrition).(16)

6. Based on the data published in specialist literature, the following molecular markers were selected for the immunofluorescence assays.

- EGFR and HER2/neu as oncogenes;
- p53 and APC as tumor suppressor genes;
- p53 is a gene whose proteic product is involved in the control process of the cellular cycle and it proved to be very useful for the identification of the genetic modifications on the level of the cellular cycle;
- Bcl2 was singled out for the assessment of the apoptotic capacity of the cells in the different stages of the esophagealtumor.

7. In the investigated cases of squamous esophagus cancer there has been highlighted supraexpression of EGFR and HER2, as compared to the normal tissue specimens.

8. The patients in whom the supraexpression of these types of oncogenes have been detected could become potential candidates for anti-cancer treatment with molecular targets (for instance anti-EGFR medication).

9. The p53 supraexpression detected in the cases investigated by means of immunofluorescence can be related to the prognosis, degree of differentiation, degree of proliferation, and it is sustained by the number of immunomarked cells and by the intensity of the marking.

10. We are in a position to conclude that, for the investigated cases of malignant esophagealtumors, which featured different degrees of differentiation and different invasion stages, we could not find any Bcl2 expression since, after setting in of malignancy, the cells acquire mechanisms of preventing apoptosis.

11. The assessment methods for the genetic expression immunofluorescence can prove to be useful in the determination and/or confirmation of the diagnosis of tumors present in the gastro-intestinal tract and, at the same time, they can be used also for selecting and directing the appropriate therapeutic strategies.

12. The MLPA method has been applied for the investigation of certain possible correlations between the previously selected gene of interest - BRCA2, and the presence of esophagus cancer.

13. Following these investigations there have been discovered certain regions belonging to these genes, which might be correlated with the esophagus cancer

14. In cause is the existence of certain deletions in the exons 4 and 5 belonging to the gene BRCA2, encountered in all 5 patients investigated for these gene, of some deletions in the exons 23, 24 and 27A, encountered in 3 of the 5 investigated patients, and of certain amplifications in the exons 8 and 10 in 3 of our patients.

15. The mutations in the gene p53 (polymorphisms, deletions) count among the most common genetic modifications discovered in human cancer cases.

16. The missense mutations produced in vivo may lead to substitutions of one amino acid only, which may in turn result in changes of the p53 protein conformation, when the substituted amino acid is replaced by another amino acid with a different electric charge.

17. In the investigated patients there have been identified point mutations in the exon 5, codon 175, in the exon 6, codons 213 and 220, and in the intron 7 in the positions +18437, T/C and +18457, T/G.

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